THE ROLE OF miR-143 AND miR-145 IN THE INVASION OF GLIOBLASTOMA

A Senior Scholars Thesis

by

MATTHEW KEITH RONCK

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Approved by:

Research Advisor: L. Gerard Toussaint
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

The Role of miR-143 and miR-145 in the Invasion of Glioblastoma.
(April 2010)

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Glioblastoma multiforme (GBM) is the most common and most malignant type of primary brain tumor. It is highly invasive and therefore difficult to treat. The life expectancy of patients harboring GBM is around 12-18 months, even in the best clinical trials. GBM invasion prevents a surgical cure; by the time the diagnosis is made, tumor cells have invaded normal tissue remote from the tumor mass. Small noncoding RNAs may contribute to the invasive phenotype of GBM. MicroRNAs (miRNAs) are small, single stranded noncoding regulatory RNA molecules that function to modulate the activity of specific mRNA targets and play important roles in a wide range of physiological and pathological processes. A previously established *in vitro* method was used to create GBM sub-populations with enhanced invasion (IM3 subpopulations). Comparison of micro-RNA expression profiles between GBM parental cell lines and IM3 sub-populations revealed differentially expressed miRNAs between the two cell lines. Two of these miRNAs, miR-143 and mIR-145 were found to be largely over-

expressed in the IM3 subpopulations and may serve as potential mediators of the invasive phenotype. Knockdown of these miRNAs in U87 cell lines showed an alteration in GBM invasion. These miRNAs may serve as therapeutic targets that decrease tumor invasion.

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I would like to personally acknowledge and thank Dr. Gail S. Martin and Dr. Sun Wu Koo for being incredibly patient with me and for helping me figure out how to perform research on a professional level. I would also like to especially thank Dr. L. Gerard Toussaint, not only for allowing me the opportunity to work in a research lab as an undergraduate research student, but also for inspiring me to see beyond just the clinical side of medicine and to focus as well on the basic science behind it.

NOMENCLATURE

FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma
IM3	Invaded through Matrigel® three times
LNA	Locked nucleic acid
miRNA	Micro-RNA
RNA	Ribonucleic acid
siRNA	Small interfering RNA

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CHAPTER I

INTRODUCTION

Glioblastoma (GBM) is the most common and most invasive type of primary brain tumor. It affects patients of all ages, but accounts for 56% of all brain tumors from ages 0-14. This tumor has proved incredibly difficult to treat. Several treatment modalities include complete surgical resection, external beam radiation, and aggressive chemotherapy (Giese et al., 2003); all have proven palliative, resulting in a dismal survival rate.

MicroRNAs (miRNAs) are a newly recognized class of micromolecules whose role in the body has only recently begun to be studied and understood. miRNAs are short (18-22 nucleotides), noncoding, single-stranded RNA molecules that function to down-regulate gene expression post-transcriptionally by binding to the 3' untranslated regions of target mRNAs. In the last several years, it has been shown that miRNA expression is frequently disregulated in cancer. For example, some miRNAs have been shown to up or down regulate different proteins central to the cell cycle, permitting tumorigenesis. Several miRNAs are over or under-expressed in glioma cells, affecting a wide range of processes including cellular proliferation, angiogenesis, differentiation,invasion, and

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This thesis follows the style of The Journal of Neuroscience.

apoptosis (Silber et al., 2009). For example, a major miRNA regulator of glioma, miR-21, has been shown to target a number of pathways leading to tumorigenesis, including components of the p53, TGF-beta, and mitochondrial apoptosis tumor-suppressor pathways. It has even been shown to promote chemotherapy resistance (Papagiannakopoulos et al., 2008). Additionally, miR-21 promotes invasion in glioma cells by downregulating matrix metalloproteinase inhibitors, leading to invasion of glioma cells (Gabriely et al., 2008). miR-143 and miR-145, the subjects of this experiment, have already been linked to tumor invasion. miR-143 has been identified as a mediator of hepatocarcinoma metastases by repressing fibronectin expression (Zhang et al., 2009), and both miR-143 and miR-145 are thought to play a large role in colorectal cancers (Wang, et al. 2009). Despite all of this, a knowledge gap exists in regard to miRNA regulation of GBM invasion. Little is known about the mechanisms of how this tumor produces such highly invasive cells. miRNAs could be key contributors to the overall invasive phenotype of GBM cells.

In order to determine which miRNAs serve as regulatory elements in GBM invasion, highly invasive GBM subpopulations were produced via serial selection through a simple, previously established *in vitro* method (Toussaint et al., 2007) utilizing a Boyden Chamber (see Figure 1). The Boyden Chamber consists of an upper and lower chamber

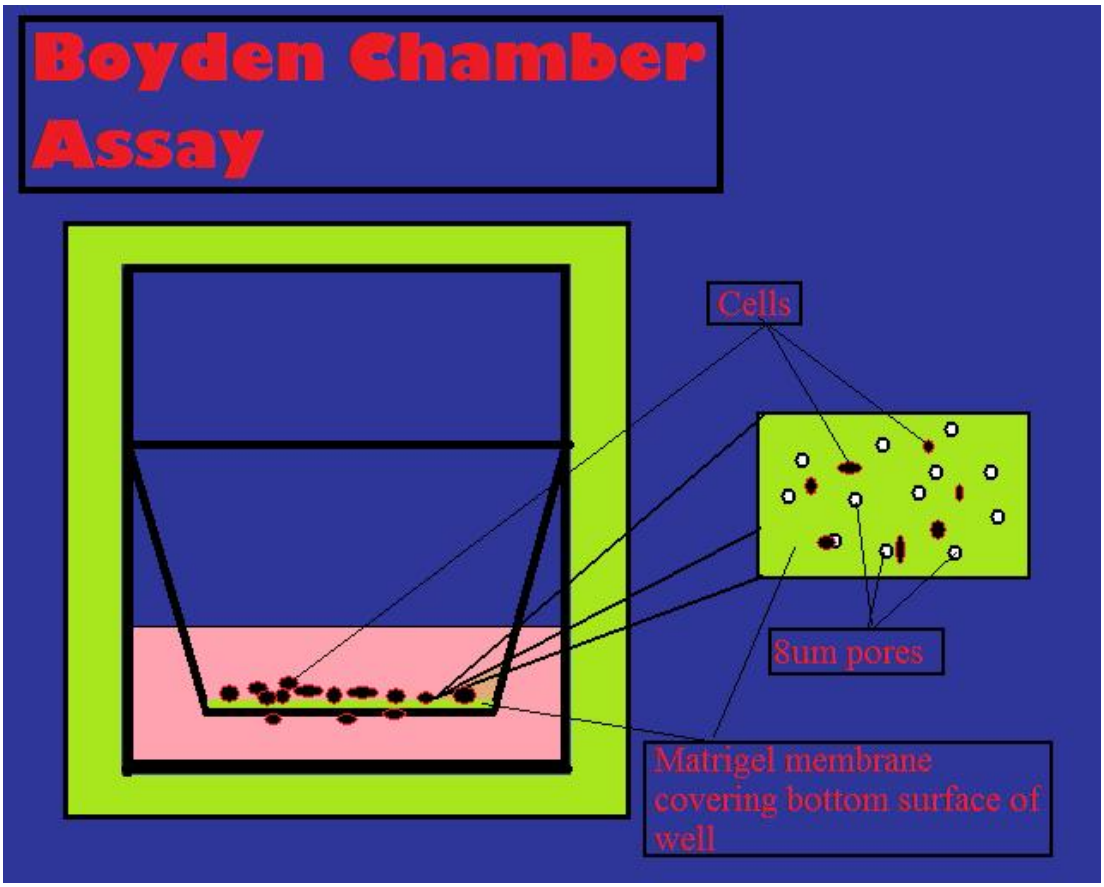
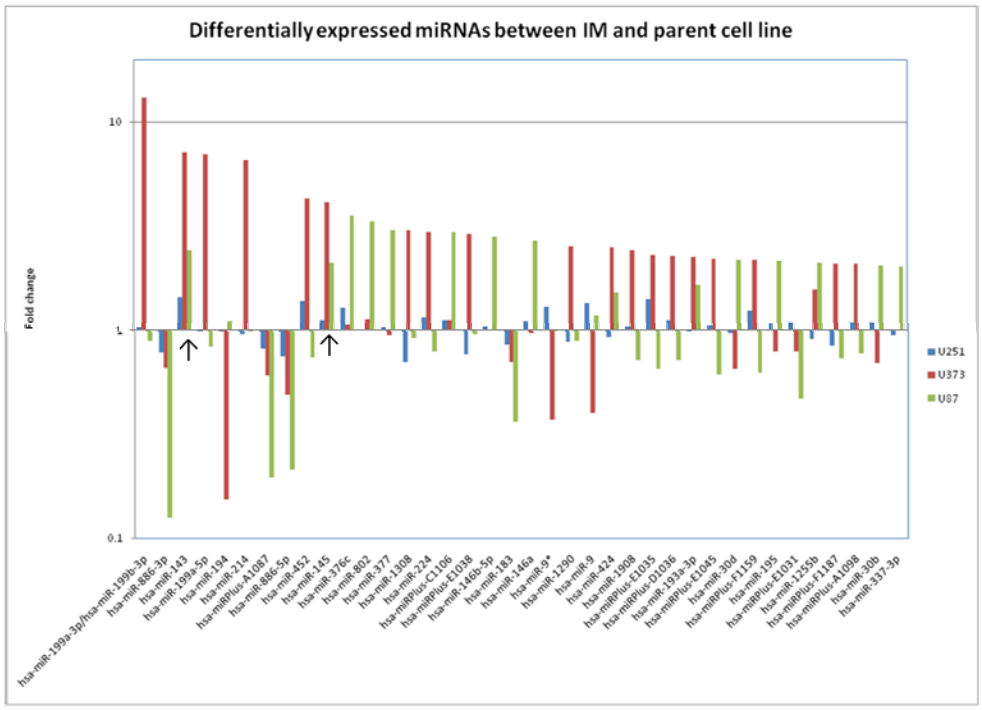


Figure 1: Boyden Chamber Invasion Assay. U87 and U87IM3 cells are pipetted into the upper chamber and left alone for a set amount of time. In order to invade into the lower chamber, cells must chew through a Matrigel® membrane and squeeze through microscopic, 8um pores. After 21 hours, only cells on the lower, outer face of the well are counted.

which are separated by a plastic wall with numerous, randomly distributed 8um pores. Matrigel®, a gelatinous protein material that is secreted by rat sarcoma cells, coats the horizontal dividing wall of the chamber. In this case, Matrigel® is used as a rough *in vitro* approximation of brain tissue. This substance is coated along the upper dividing wall of the chamber and allowed to settle into the 8um pores. GBM cells are seeded at a specific density in the upper chamber, and FBS (Fetal Bovine Serum) is placed in the

lower chamber. FBS acts as a chemoattractant for the GBM cells. In order for invasion into the lower chamber to occur, highly invasive cells must chew up the Matrigel® and squeeze through these microscopic holes, leaving cells that are less invasive in the upper chamber. After a set amount of time, these highly invasive cells are harvested and collected from the bottom chamber and are hence called the IM cell lines (Invaded through Matrigel®). Invasive subpopulations that have invaded through Matrigel® three times are thus called IM3 subpopulations and are considered to be unique subpopulations of a highly invasive nature. These IM3 subpopulations are used as a basis for comparison against parental (normal GBM) cell lines for subsequent siRNA knockdown experiments. This IM3 cell selection was a previously established method (Toussaint et al., 2007).

RNA profiling data was generated and compared between the parental and the IM3 subpopulations for three GBM cell lines, U87, U373, and U251. Based on this data, a list was generated of potential miRNA targets that regulate GBM invasion (see Figure 2). Of these, miR-143 and miR-145 were shown not only to be largely upregulated in the IM3 cell type of all cell lines, but their graphs were closely similar in shape and amplitude, suggesting a similar regulatory mechanism driving the expression of both. This led to the hypothesis that these two specific miRNAs play a large regulatory role in GBM invasion.



Upregulated in Invasive (IM3) Cells?

	Total RNA fraction	Small RNA fraction (not shown)
miRNA-143:	U87, U251, U373	U87, U373, C6
miRNA-145:	U87, U251, U373	U87, U373

Figure 2: Selection of miRNA Candidates. miRNA analysis of three human GBM cell lines revealed many over and under-expressed miRNAs between the parental and highly invasive subpopulations. Small fraction was not included because that fraction is apt to produce errors with smaller RNAs. These 38 candidates are ones that express a fold change >2 between the two cell lines. Note that a fold change of >1 correlates to miRNAs that were overexpressed in the IM3 subpopulations. Of these candidates, miRNA-143 and miRNA-145 showed the greatest unidirectional, positive fold change in all cell lines (arrows in figure).

Antagomirs (siRNA) are short RNA sequences that are closely or exactly complementary in sequence to specific miRNA targets. Antagomirs can be transfected into cells in order to act via a pathway call RNA silencing. In this pathway, antagomirs bind a specific miRNA in the cell, leading to its destruction or inhibition. This, in turn, prevents the miRNA from inhibiting mRNA expression. In this experiment, knockdowns

were performed on both parental and IM3 cell lines using specific antagomirs of miRNA-143 and miRNA-145 (anti-miR-143 and anti-miR-145, respectively), which bind to and individually inhibit miR-143 and miR-145 miRNA function. Several different combinations of knockdown experiments were performed using these antagomirs. IM3 cells and parental GBM cells were transfected with knockdown probes, run in a Boyden Chamber assay against a parallel control, and compared against each other via total cell counts.

Attachment is a characteristic frequently altered in tumor cells. In order for cells to invade through to the lower chamber, they must first attach to the Matrigel® membrane. It is important to be able to rule out any major alterations in attachment ability caused by the antagomir knockdowns because alterations in attachment can give skewed invasion results. In order to account for this, knockdown antagomir-transfected cells were run through attachment assays to illustrate any alteration of cellular attachment ability.

miR-143 & miR-145 knockdown in U87 and U87IM3 cell lines showed an alteration of the invasive phenotype in GBM cell lines, suggesting that these miRNAs do in fact play a regulatory role in GBM invasion.

CHAPTER II

MATERIALS AND METHODS

Cell lines and culture conditions

Human U87 (glioma), U251 (glioma), and U373 (astrocytic tumor) cell lines were obtained from the American Type Culture Collection (Manassas, VA.). Cells were grown in DMEM (Cellgro Media Tech) with 10% FBS, penicillin (10 IU/mL), and streptomycin (10 ug/mL) in a humidified 37˚C chamber with 5% CO_2.

RNA isolation

Total cellular RNA was extracted using the *mir*Vana miRNA Isolation kit (Ambion) per manufacturer's instructions for total RNA isolation. RNA was analyzed by miRCURY™ LNA Array microRNA Profiling Services.

Anti-miRNA transfection of U87 & U87IM3

U87 and U87 IM3 cells were transfected with either FITC-labeled anti-miR-143, anti-miR-145, or a control LNA knockdown probe which binds nothing in the cell (Exiqon, Vedbaek, Denmark) at final concentration of 50nM using Lipofectamine 2000 per manufacturer's instructions (Invitrogen, Carlsbad, CA). Culture medium was changed 8 hours after transfection and replaced with Optimem medium (Invitrogen Carlsbad, CA) for overnight recovery. Efficiency of transfection was assessed using fluorescence microscopy.

Attachment assay of U87 and U87IM3 cell lines transfected with anti-miR-143 and anti-miR-145

Wells of 24-well tissue culture plate were coated with Matrigel® Basement Membrane Matrix (BD Biosciences) diluted with DMEM (1:10) and allowed to sit for 2 hours at room temperature, after which unbound Matrigel® was aspirated. U87 and U87IM3 cells (each twice transfected separately with anti-miR-143 and anti-miR-145) in DMEM with 10%FBS were plated at 20,000 cells/mL into wells and incubated at 37˚C for 4 hours. Wells were gently washed with PBS and the remaining attached cells were fixed in 4% paraformaldehyde and stained with hematoxylin for 2 minutes. Wells were washed with PBS and cells were counted.

Boyden chamber invasion assay of U87 & U87IM3 cell lines transfected with knockdown anti-miRNA

U87 and U87IM3 cells at 300,000 cells/mL in 0.5mL DMEM (without FBS) were plated in the Matrigel®-coated upper chamber of an insert (24-well insert, pore size 8um, BD Biosciences). DMEM with 10% FBS located in the lower chamber served as the chemoattractant. Cells were allowed to invade for 21 hours at 37˚C, after which time cells on the top inner face of the wells were vigorously scrubbed off and cells remaining on the bottom layer that migrated through the Matrigel® were fixed in 4% paraformaldehyde, stained with hematoxylin, and counted.

Statistical analysis

Data are presented as mean +/- standard deviation. For parental and IM-3 comparisons, a t-test was used to determine statistical significance. A t-test with a value of $P < 0.05$ was considered significant.

CHAPTER III

RESULTS

Selection of miRNA candidates yielded two key miRNAs of interest: miR-143 & miR-145. These miRNAs were both overexpressed in the highly invasive subpopulations of all GBM cell lines, indicating a possible contribution to the invasive phenotype of glioblastoma. Not only were the fold changes all in the same direction, but it was noticed (from Figure 2) that the ratios of expression levels between the two miRNAs are very similar, which suggests a common regulatory pathway. Total RNA isolation, rather than a size-fractionation protocol, was used to generate the substrate for expression analysis.

Commercially available antagomirs were used for miR-143 and miR-145 (anti-miR-143 & anti-miR-145) knockdown. These locked nucleic acid molecules (LNA) are resistant to RNAses, and are tagged with a FITC label, which allows visualization under green fluorescence microscopy. Transfection of these antagomirs into GBM cells was verified using this form of microscopy. U87 and U87IM3 cells were each transfected separately with miR-143 and miR-145 for a total of four individual transfections (see Table 1).

Table 1: Transfection table.

	U87	**U87IM3**
miR-143	U87 w/anti-miR-143	U87IM3 w/anti-miR-143
miR-145	U87 w/anti-miR-145	U87IM3 w/anti-miR-145

Cells transfected with specific antagomirs were each run individually through a Boyden Chamber Invasion Assay. After a 21-hour invasion time, only cells that invaded through

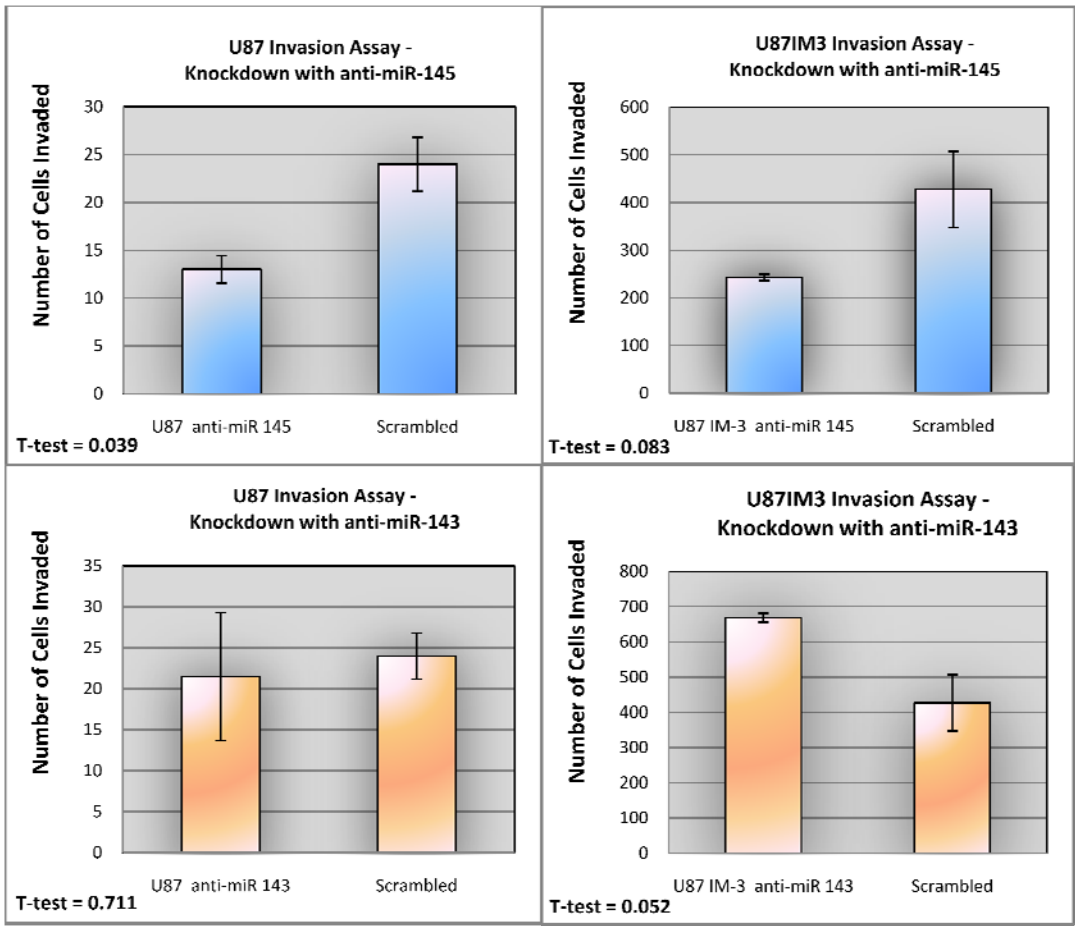


Figure 3: Invasion Assay Cell Counts. Shown are all U87 and U87-IM3 cell counts after invasion assays with cells containing knockdown probes miR-143 and miR-145. Knockdowns were run in parallel against cells transfected with a control "scramble" probe, which is a probe of a specific sequence that does not bind to anything in the cell, and thus, should have no effect on cell migration.

the Matrigel® and the 8um pores were counted (Figure 3). Four invasion assays were performed, one for each of the four transfections listed in Table 1. Knockdown of miR-145 was shown to result in decreased invasion, while knockdown of miR-143 actually increased tumor invasion. Between the two subpopulations, counts revealed that knockdown with anti-miR-145 averaged a 45% *decrease* in overall cell invasion while knockdown using anti-miR-143 averaged a 44% *increase*. Resulting cell counts were analyzed statistically and compared using a statistical t-test.

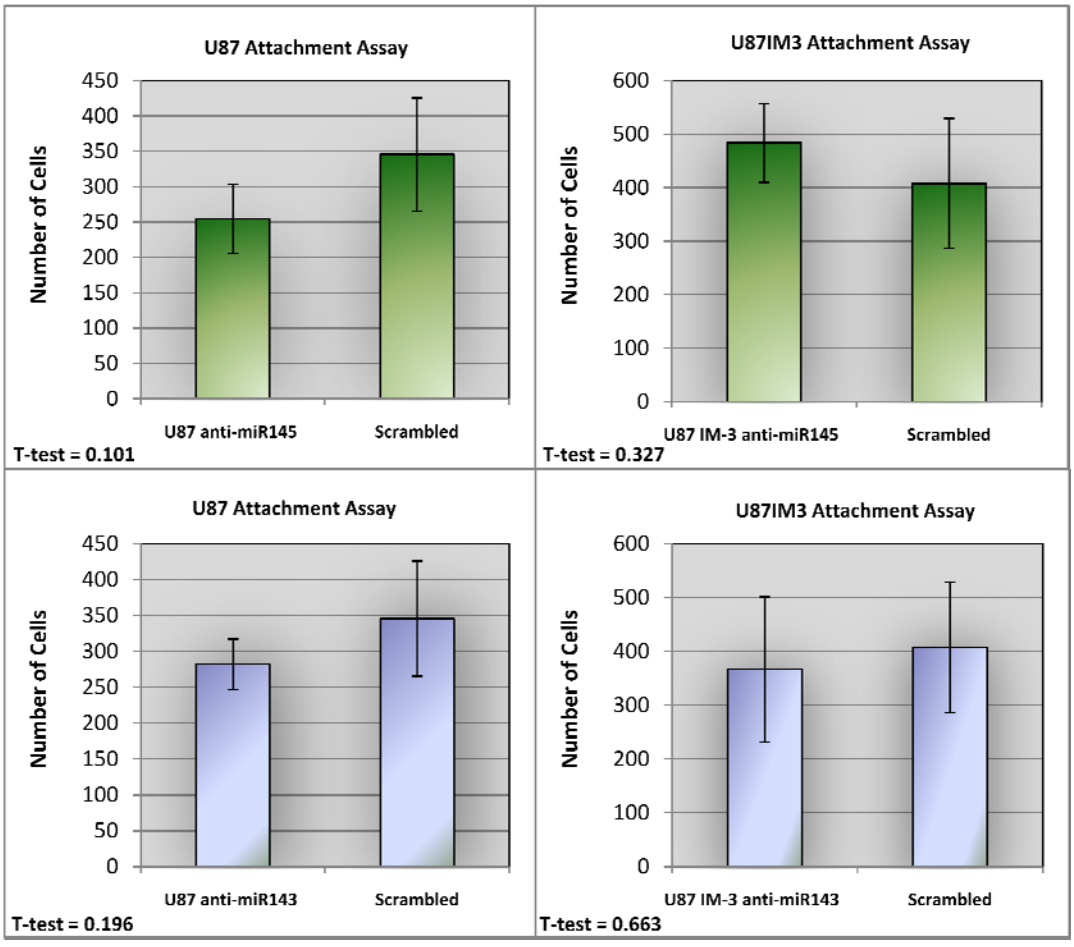


Figure 4: Attachment Assay Cell Counts. These are control assays used to account for altered attachment ability of GBM cells due to antagomir knockdown. They show that, overall, these antagomir probes exert their effects primarily on the invasive abilities of cells, and do not contribute in large part to extracellular attachment.

The attachment assays did not reveal any statistically significant alterations of attachment ability (Figure 4).

CHAPTER IV

SUMMARY AND DISCUSSION

The Matrigel®-filled Boyden Chamber allows the creation of an environment similar to the brain tissue in which GBM resides. We can utilize this environment to perform representative experiments that we could not normally do in human patients. While this setup does allow experimentation in regards to tumor invasion, it also allows the conduction of studies relating to tumor cell attachment, another commonly disregulated feature of cancer.

The attachment assays were an important addition to this experiment because attachment ability can act to mask invasive ability. Differences in attachment to Matrigel® can give one cell line a starting advantage over another when comparing their relative ability to invade through this matrix of proteins. Therefore, it is important to be able to rule out alteration of cellular attachment ability as a contributing factor of invasion. Although there were minor differences in the attachment ability of cell lines treated with antagomir knockdown, these differences were not statistically significant, nor did they account for the differences in cell invasion that we noticed.

In regards to the invasion experiments (Figure 3), the results from the miR-145 knockdown were the most promising. Invasion of these cells was reduced by almost half in the parental and IM3 cell lines. However, knockdown of miR-143 yielded different

results. Although the knockdown of the parental cells showed a decrease in invasion, the IM3 knockdowns of miR-143 actually yielded an *increase* in tumor invasion. This is confounding because both miR-143 and miR-145 seem to exhibit the same pattern of up-regulation across all cell lines (from data from the RNA isolation experiment). Thus, if miR-145 knockdown decreased invasion, it would be expected that miR-143 knockdown would do the same. This is evidently not the case. One possible explanation for this occurrence could be that these two miRNAs act along the same pathway, and their full function is only observed when the two are allowed to interact at the same time. In these experiments, knockdowns were performed in mutual exclusion by altering expression of only one miRNA at a time. A logical future experiment would include a double-knockdown of miR-143 and miR-145 at the same time. If a large overall decrease was observed, this would be strongly indicative that these two miRNAs do in fact act along a common pathway.

Although miR-143 knockdown was not beneficial towards decreasing tumor invasion, the sizeable reduction of invasion in the knockdown of miR-145 does indicate that miR-145 is a key regulator of the highly invasive phenotype of GBM. Concluding this experiment, future plans include *in vivo* experiments to support the hypothesis that miR-145, perhaps in combination with miR-143, plays a significant role in GBM tumor invasion. Eventually, it is hoped that, through testing, a treatment for human patients with GBM can be developed. While this form of treatment may not be a panacea

for GBM, it may help to slow down tumor invasion and increase life expectancy.

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